THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 49, Issue of December 3, pp. 35147–35151, 1999
Printed in U.S.A.

Mammalian HSP60 Is a Major Target for an Immunosuppressant Mizoribine*

(Received for publication, April 12, 1999, and in revised form, September 7, 1999)

Hideaki Itoh‡§, Atsushi Komatsuda¶, Hideki Wakui¶, Akira B. Miura¶, and Yohtalou Tashima‡

From the ‡Department of Biochemistry and ¶Third Department of Internal Medicine, Akita University School of Medicine, 1-1-1 Hondo, Akita City 010-8543, Japan

It has been reported that immunosuppressant cyclosporin A or FK506 binds to immunophilins in the cell and that these immunophilins make a complex with molecular chaperones HSP70 or HSP90. Although mizoribine has been used clinically as an immunosuppressant, immunophilins of the agent have not yet been fully understood. We investigated their specific binding proteins using mizoribine affinity column chromatography and porcine kidney cytosols. By increasing mizoribine in the eluant from the column, two major proteins (with molecular masses of 60 and 43 kDa) were detected by SDS-polyacrylamide gel electrophoresis. Based on the amino acid sequence analysis of these proteins, 60- and 43-kDa mizoribine-binding proteins were identified with HSP60 and cytosolic actin, respectively. A considerable amount of actin was also eluted from the affinity column by nucleotides, but a very low quantity of HSP60 was eluted under the same conditions. On the other hand, HSP60 was eluted as a major protein in the eluant that was eluted preferentially, with nucleotide followed by mizoribine. Actin was also detected in the eluant, but the quantity of the protein was very low. These results indicated that HSP60 has high affinity to mizoribine, and the interaction was also observed on surface plasmon resonance analysis. Although HSP60 or GroE facilitated refolding of citrate synthase *in vitro*, mizoribine interfered with the chaperone activity of HSP60. On different types of mizoribine affinity columns, HSP60 or actin recognized the NH_2 group of mizoribine, and this group may be a functional group of the agent.

Immunosuppressant cyclosporin A or FK506 binds to specific binding proteins and performs an immunosuppressive function in the cells. These immunosuppressant-specific binding proteins are called immunophilins. Some immunophilins bind to molecular chaperones HSP70 and HSP90 (for review, see Ref. 1). It has been shown that the immunophilins of cyclosporin A are cyclophilins-A–D, cyclophilin-40, actin, and HSP70 (for review, see Ref. 1). The immunophilins of rapamycin and FK506 are the same and they are FKBP12, FKBP13, FKBP25, and FKBP52 (2). In these immunophilins, FKBP52 binds to the 90-kDa molecular chaperone HSP90, and the complex is involved in steroid hormone receptors (3). On the contrary, HSP70 and HSP90 bind directly to an immunosuppressant deoxyspergualin (4, 5). HSP70 and HSP90 themselves may act as immunophilins for the reagent.

Mizoribine (4-carbamoyl-1-β-D-ribofuranosylimidazolium-5-olate) is a purine nucleotide analog isolated from *Eupenicillium brefeldianum* (6) and has been shown to exhibit cytotoxic effects in mammalian cell culture studies (7). On the basis of these reports, mizoribine has been used clinically as an immunosuppressant after renal transplantation (8), for lupus nephritis, and recently for rheumatoid arthritis and Behçet's disease (9). The mechanisms of the immunosuppressive effects of mizoribine demonstrated that mizoribine is phosphorylated by adenosine kinase (10), and the monophosphate inhibits from IMP dehydrogenase (10) or both IMP dehydrogenase and GMP synthase (11). The inhibition of the enzymes results in the depletion of intracellular guanine nucleotides as shown in both murine and human systems (12).

There are many reports on drug evaluation or the clinical effects of mizoribine, but no report has been shown for mizoribine-specific binding proteins. Until now, the immunophilins of mizoribine have not yet been understood. In the present study, we have tried to search mizoribine-binding proteins using mizoribine affinity columns prepared on different types of affinity gels. We identified two proteins, with molecular masses of 43 and 60 kDa, and will discuss their physiological implications and possibilities of their function.

EXPERIMENTAL PROCEDURES

Materials—Porcine liver HSP60 or bovine brain HSP70 was purified, and an antibody against each HSP was produced as described previously (13, 14). GroEL and GroES were from StressGen. Pig heart citrate synthase was from Roche Molecular Biochemicals.

Mizoribine Affinity Column Chromatography—An immunosuppressant mizoribine (4-carbamoyl-1-β-D-ribofuranosylimidazolium-5-olate) was obtained from Asahi Chemical Industry Co., Ltd. (Tokyo, Japan). The chemical constitution of mizoribine is shown in Fig. 1. Mizoribine-Sepharose was prepared using mizoribine and different types of affinity gels, activated epoxy-Sepharose 6B, CNBr-activated Sepharose 4B, or activated CH-Sepharose 4B (Amersham Pharmacia Biotech) according to the instruction manual. Porcine kidneys (200 g) were homogenized with three volumes of 10 mM Tris-HCl (pH 7.4) and centrifuged at 20,000 × *g* for 20 min at 4 °C. The supernatant was collected and followed by centrifugation at 105,000 × *g* for 60 min at 4 °C. The supernatant obtained from ultracentrifugation was used as porcine kidney cytosol in the present study. Porcine kidney cytosols were applied to the column, equilibrated in the same buffer, and washed with 20 column volumes of the buffer containing 0.15 M NaCl. After washing the column, binding proteins were eluted with a linear gradient of mizoribine (0–10 mM in 10 mM Tris-HCl, pH 7.4). The binding proteins were also eluted from the columns with nucleotides. 5 mM AMP, 5 mM ATP, 5 mM GTP, or 10 mM mizoribine. Otherwise, proteins were eluted preferentially, with nucleotide followed by mizoribine; 5 mM AMP followed by 10 mM mizoribine, 5 mM ATP followed by 10 mM mizoribine, or 5 mM GTP followed by 10 mM mizoribine. 50% trichloroacetic acid solution (w/v) was added to each fraction at a final concentration of 5% (w/v) and kept on ice for 30 min. The precipitates were collected by centrifugation at 20,000 × *g* for 20 min at 4 °C. The precipitates were

* This work was supported in part by Grant-in-aid for Scientific Research (priority areas of molecular chaperone: 09276201 and 10172201 to H. I.; encouragement of young scientists: 09770840 to A. K.; and C2: 11671024 to H. W.) from the Japanese Ministry of Education, Science, Sports and Culture. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed. Tel.: 81-18-884-6078; Fax: 81-18-836-2606; E-mail: hideaki@med.akita-u.ac.jp.

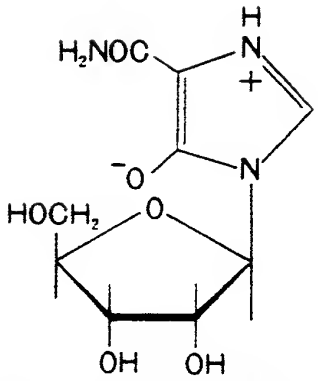

FIG. 1. **Chemical constitutional formula of mizoribine (4-carbamoyl-1-β-D-ribofuranosylimidazolium-5-olate).** Haworth projections of the structure are shown.

analyzed by SDS-PAGE[1] (12.5% gel) (15), stained with 0.1% Coomassie Brilliant Blue (w/v), and destained with 25% isopropyl alcohol (v/v) and 10% acetic acid (v/v) or by immunoblotting (16) using an anti-HSP60 antibody and alkaline phosphatase anti-rabbit IgG antibody (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD).

Amino Acid Sequence of the Mizoribine-binding Proteins—Mizoribine-binding proteins were electrophoresed by SDS-PAGE (12.5% gel). After staining the gel with 0.1% Coomassie Brilliant Blue (v/v) and destaining with 25% isopropyl alcohol (v/v) and 10% acetic acid (v/v), protein bands were excised and digested using lysyl endopeptidase (Wako Pure Chemical Industries; Osaka, Japan) as described previously (13, 17). The peptides were purified by the reverse phase column that was connected to an HPLC apparatus. A column of Wakopak (Wakosil $5C_{18}$; Wako Pure Chemical Industries; Osaka, Japan) was connected to an HPLC apparatus (Amersham Pharmacia Biotech high performance liquid chromatographer equipped with two model 2150 pumps, a model 2150 HPLC controller, and a model 2158 Uvicord SD UV detector was used for purification of the peptides). The peptide was applied onto the column, which was pre-equilibrated with 0.1% trifluoroacetic acid (v/v) and eluted with a linear gradient of 0–64% acetonitrile (v/v) in 0.1% trifluoroacetic acid (v/v) at a flow rate of 0.5 ml/min, and 0.5-ml fractions were collected. The purified peptides were detected at 206 nm as described previously (13, 17). Amino acid sequencing of the purified peptides was performed with 491 Procise® protein sequence system (Perkin-Elmer).

Real Time Analysis of HSP60-Mizoribine Interaction by Surface Plasmon Resonance—The BIAcore apparatus was from BIAcore (Uppsala, Sweden). All experiments were carried out at 25 °C. Porcine liver HSP60 or GroEL was covalently linked to the surface of a flow cell on a research grade CM5 sensor chip using an amine-coupling kit from BIAcore (Uppsala, Sweden). The flow was 5 μl/ml throughout the derivatization procedure. The derivatized sensor chip was washed extensively with HBS-P buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 0.005% surfactant P20). The binding kinetics of mizoribine to immobilized HSP60 was determined by increasing mizoribine (0.15625–2.5 μM) in HBS-P buffer (running buffer) at a flow rate of 20 μl/ml. The sensor chip surface was regenerated by 1.5 M NaCl in HBS-P buffer at a flow rate of 20 μl/ml. Unspecific binding was evaluated using flow cells on the chip with either underivatized surface or surface derivatized with bovine brain HSP70, because HSP70 had no interaction with mizoribine during this analysis. The kinetic parameters of the binding reactions were determined using BIAevaluation version 3.0 software. The dissociation rate constant (k_d) was determined from a plot of $\ln(R_0/R)$ *versus* time, with R being the surface plasmon resonance signal at time t; the association rate constant (k_a) was determined from a plot of $\ln(\mathrm{abs}(dR/dt))$ *versus* time. The apparent equilibrium dissociation constant was calculated from the kinetic constant $K_D = k_d/k_a$.

Chaperone Activity of HSP60—Chaperone activity of mammalian HSP60 or GroEL/GroES was measured using citrate synthase as described (18, 19). Briefly, citrate synthase was denatured (at a concentration of 15 μM) in a buffer containing 6 M guanidine hydrochloride, 100 mM Tris-HCl, pH 7.4, 2 mM EDTA, 20 mM dithiothreitol. Renaturation was initiated by diluting the denatured citrate synthase 100-fold into a buffer containing 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 10 mM KCl, and 2 mM ATP at 20 or 35 °C. Details for individual experiments are given in the legend to Fig. 7.

[1] The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; IMPDH, inosine-monophosphate dehydrogenase.

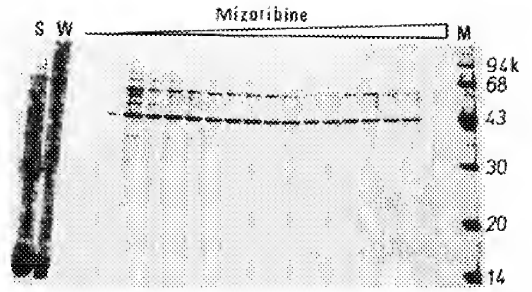

FIG. 2. **Mizoribine affinity column chromatography.** The mizoribine affinity column was prepared using mizoribine and activated epoxy-Sepharose 6B as described under "Experimental Procedures." Porcine kidney cytosols were applied onto the column, and after washing the column with a large amount of the buffer containing 0.15 M NaCl, proteins were eluted with a linear gradient of mizoribine (0–10 mM), and the eluants from the column were electrophoresed by SDS-PAGE (12.5% gel) followed by staining with 0.1% Coomassie Brilliant Blue (w/v) and destaining with 25% isopropyl alcohol (v/v) and 10% acetic acid (v/v). *S*, *W*, and *M* denote applied samples, washed proteins, and molecular standard proteins, respectively.

RESULTS

Mizoribine Affinity Column Chromatography—To search mizoribine-binding proteins, we analyzed them using a mizoribine affinity column (prepared using activated epoxy-Sepharose 6B). Porcine kidney cytosols were applied onto the column, and a thorough washing with a buffer containing 0.15 M NaCl was carried out to avoid nonspecific binding proteins. After washing, the binding proteins were eluted with a linear gradient of mizoribine (0–10 mM), and eluted proteins were detected by SDS-PAGE. We detected two major protein bands (with molecular masses of 60 and 43 kDa) on the gel (Fig. 2). Some minor proteins (with molecular masses of 40, 50, 55, and 80 kDa) were also detected by faint bands on the gel. In the present study, we focused on these two major proteins.

Amino Acid Sequence of the Mizoribine-binding Proteins—To identify the mizoribine-binding proteins, we investigated the amino acid sequence of these two proteins using a protein sequencer. Next the proteins were electrophoresed by SDS-PAGE, and the protein bands were excised and digested with lysyl endopeptidase. The digested peptides were purified using a C_{18} reverse phase column connected to an HPLC. Fig. 3*A* shows a peptide map obtained from the 60-kDa kidney protein that was eluted from a mizoribine affinity column. One peptide (fraction 60) was sequenced by a protein sequencer. The sequence contained three peptides, and the 35 amino acid residues obtained from the peptide (fraction 60) completely coincided with human HSP60, the amino acid sequence of which was deduced from the nucleotide sequence of the cDNA (20). Fig. 3*B* shows the sequence obtained from the peptide and the position of human HSP60. On the basis of molecular mass and amino acid sequence data, HSP60 with a molecular mass of 60 kDa was one of the major binding proteins to the mizoribine affinity column.

We also investigated the amino acid sequence of another 43-kDa mizoribine-binding protein. Fig. 4*A* shows a peptide map of the protein. The two peptides (fractions 57 and 66) were sequenced, and 15 amino acid residues were obtained from each peptide (Fig. 4*B*). The sequence of these peptides has complete homology to human cytoplasmic actin (G-actin) (21). These results indicated that the 43-kDa mizoribine-binding protein is actin.

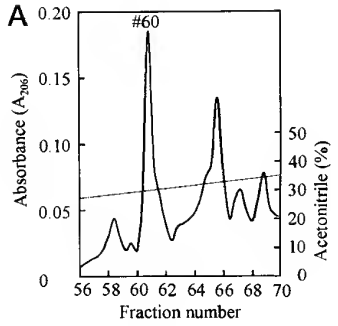

FIG. 3. **HPLC fractionation of lysyl endopeptidase digests and the amino acid sequence data of the 60-kDa mizoribine-binding protein.** *A*, lysyl endopeptidase digests of the 60-kDa kidney proteins were separated by reverse phase chromatography on a C_{18} column with a linear gradient of 0–64% acetonitrile (v/v) in 0.1% trifluoroacetic acid (v/v) at a flow rate of 0.5 ml/min. *B*, the purified peptide indicated in the panel (*#60*) was sequenced by a peptide sequencer. *Numbers* in *parentheses* indicate the position of human HSP60.

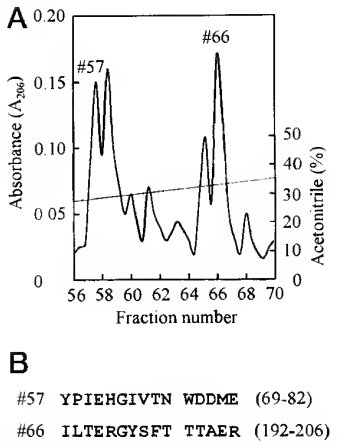

FIG. 4. **HPLC fractionation of lysyl endopeptidase digests and the amino acid sequence data of the 43-kDa mizoribine-binding protein.** *A*, lysyl endopeptidase digests of the 43-kDa kidney proteins were separated by reverse phase chromatography on a C_{18} column with a linear gradient of 0–64% acetonitrile (v/v) in 0.1% trifluoroacetic acid (v/v) at a flow rate of 0.5 ml/min. *B*, the purified peptides indicated in the panel (*#57* and *#66*) were sequenced by a peptide sequencer. *Numbers* in *parentheses* indicate the position of human actin.

Effect of Nucleotides on Mizoribine-binding Proteins—As shown in Fig. 1, mizoribine is a purine nucleotide analog. We investigated the influence of nucleotides on mizoribine and their binding proteins, because the chemical structures of mizoribine and the nucleotides resemble each other. In this study, we used a mizoribine affinity column prepared with activated epoxy-Sepharose 6B. Porcine kidney cytosols were applied to the column and washed with 20 column volumes of the buffer containing 0.15 M NaCl to avoid nonspecific binding proteins. After washing the column extensively, the binding proteins were eluted using AMP, ATP, GTP, or 10 mM mizoribine. The remaining proteins were further eluted by using mizoribine from each affinity column, and the eluants were analyzed by SDS-PAGE. As shown in Fig. 5*A*, many protein bands were detected in the eluants from nucleotide alone (AMP, ATP, and/or GTP). The purine structure or mono- and triphosphate of nucleotides affected slight differences in the eluted proteins.

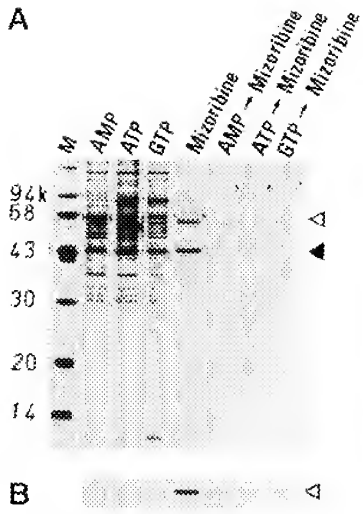

FIG. 5. **Influence of nucleotides on the mizoribine-binding proteins.** Porcine kidney cytosols were applied onto a mizoribine affinity column (prepared from activated epoxy-Sepharose 6B), and the column was washed with buffer containing 0.15 M NaCl. After washing, the binding proteins were eluted with 5 mM AMP, 5 mM ATP, 5 mM GTP, or 10 mM mizoribine. On each AMP-, ATP-, and GTP-eluted column, the remaining proteins were then eluted with 10 mM mizoribine. The eluants were analyzed by SDS-PAGE (12.5% gel), stained with 0.1% Coomassie Brilliant Blue (w/v), and destained with 25% isopropyl alcohol (v/v) and either 10% acetic acid (v/v) (*A*) or by immunoblotting using anti-HSP60 antibody (*B*). In *panel A*, *M* denotes molecular marker proteins. In both panels, the *open* and *closed triangles* indicate HSP60 and actin, respectively.

Among those with molecular masses of 80–90 kDa, proteins were eluted by ATP, but none or few of these proteins were eluted by AMP. This might be an effect of the number of phosphates, and these proteins might be eluted by ATPase. On the contrary, the 10-kDa protein was one of the major proteins eluted by GTP. The protein was detected as a faint protein band in the eluant from AMP or ATP. The protein was affected by the difference of the purine structure of the nucleotide. In the eluants, the 43-kDa protein, actin, was detected as a major protein from these nucleotides. The density of actin in these eluants was the same as in the eluant from mizoribine. However, the protein was also detected in the eluants from nucleotides followed by mizoribine as very faint protein bands on SDS-polyacrylamide gels. On the contrary, HSP60 was eluted from the affinity column by nucleotides, with a minor protein detected by immunoblotting (Fig. 5*B*). The quantity of HSP60 was highest in the eluant from mizoribine. During immunoblotting analysis, the protein bands were detected to some extent in the eluant from nucleotides followed by mizoribine, and the density of HSP60 protein bands in the eluant was higher than that of the protein bands in the eluant from nucleotides only (Fig. 5*B*). These results indicate that HSP60 has high affinity to mizoribine among these two major mizoribine-binding proteins.

Affinity Determination of the Interaction between HSP60 and Mizoribine by Surface Plasmon Resonance Analysis—A sensorgram of the interaction of an HSP60 and mizoribine is shown in Fig. 6. Using BIAcore evaluation software, the kinetic parameters of k_a, k_d, and K_D were measured. The binding curves were fitted to a simple bimolecular binding algorithm with x^2 = 0.332. The results of the surface plasmon resonance analysis with immobilized HSP60 and soluble mizoribine were k_a (1/M·s) = 1.21×10^{-5}, and k_d (1/s) = 6.67×10^{-2}. The apparent equilibrium dissociation constant (K_D) was calculated from these kinetic constants, and the K_D value obtained was 5.53 ×

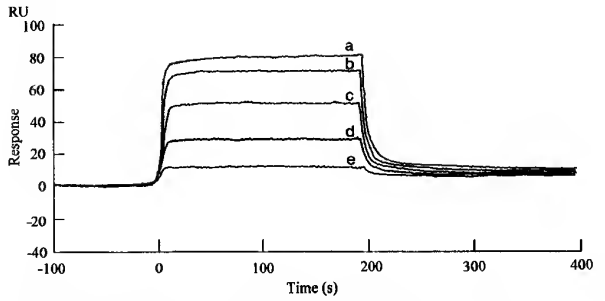

FIG. 6. **Surface plasmon resonance analysis of the interaction between HSP60 and mizoribine.** A sensorgram for the binding of HSP60 to mizoribine is shown. Different concentrations of mizoribine were injected as described under "Experimental Procedures." Traces *a–e* indicate 2.5, 1.25, 0.625, 0.3125, and 0.15625 μM mizoribine, respectively. *RU*, resonance units.

10^{-7} M. During the analysis, the same data were obtained from GroEL and HSP60 (data not shown).

Interference of Mizoribine on Chaperone Activity of HSP60—Citrate synthase refolding was analyzed under permissive conditions (20 °C), where spontaneous reactivation could occur, or under nonpermissive conditions (35 °C), where spontaneous reactivation could not occur, as described (19). As shown in Fig. 7, citrate synthase was partitioned to its native state in a chaperonin-independent folding reaction under permissive conditions. Mizoribine did not influence the reaction. On the contrary, spontaneous renaturation of citrate synthase was not observed under nonpermissive conditions. Under these conditions, the recovery of active citrate synthase was about 63% in the presence of the complete chaperonin system (including HSP60, GroES, and ATP). Mizoribine interfered with the refolding reactions of citrate synthase even in the presence of the complete chaperonin system. These results indicated that the interaction between HSP60 and mizoribine may be physiologically significant.

Functional Groups of Mizoribine—We investigated the functional groups of mizoribine for these binding proteins. In the present study, we prepared the mizoribine affinity column using three different types of affinity gel matrices (CNBr-activated Sepharose 4B, activated CH-Sepharose 4B, and activated epoxy-Sepharose 6B). In these affinity gel matrices, both CNBr-activated Sepharose 4B and activated CH-Sepharose 4B coupled with the NH_2 group of ligands. The different mechanisms of these two affinity matrices are as follows. Activated CH-Sepharose 4B has a spacer of carboxyl-hexyl chain (C6) and is coupled with the NH_2 group of ligand, but it does not have CNBr-activated Sepharose 4B. On the contrary, epoxy-activated Sepharose 6B coupled directly with the OH group of sugar or carbohydrates. As shown in Fig. 8, we investigated the proteins (that bind to mizoribine affinity gels) prepared with different types of affinity gel matrices as described above. SDS-PAGE could detect no protein bands in the eluant from all control affinity gels (Fig. 8*A*). Furthermore, protein bands were also not detected in the eluant from mizoribine affinity gels prepared with either CNBr-activated Sepharose 4B or activated CH-Sepharose 4B. On the contrary, HSP60 and actin were detected only in the eluant from the mizoribine affinity column prepared with activated epoxy-Sepharose 6B. The same data were obtained by immunoblot analysis using an anti-HSP60 antibody (Fig. 8*B*). These results indicated that HSP60 and/or actin are binding proteins to mizoribine, and they recognize the NH_2 group of mizoribine (but not OH groups of the agent). Based on these results, we determined that the NH_2 group of mizoribine may act as a functional group.

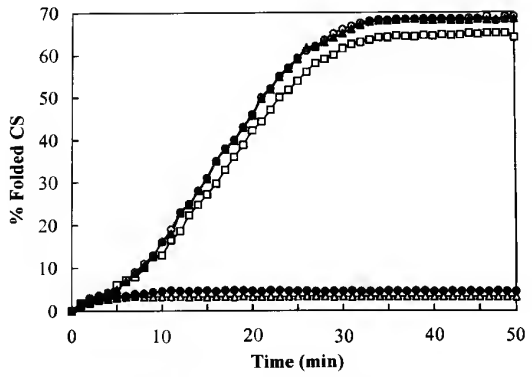

FIG. 7. **Chaperone activity of HSP60.** Chaperone activity of HSP60 was analyzed under different conditions. Denatured citrate synthase was rapidly diluted to a concentration of 150 nM into a buffer (50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 10 mM KCl, 15% glycerol (v/v)) at 4 °C. For the spontaneous folding reaction, denatured citrate synthase was diluted directly into the buffer in the presence (*open circle*) or absence (*closed triangle*) of 5 mM mizoribine pre-equilibrated to 20 °C. Under other conditions, denatured citrate synthase was rapidly diluted to a concentration of 150 nM into a buffer (50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 10 mM KCl) and 225 nM HSP60 at 4 °C. The temperature was immediately adjusted to 35 °C, and the following additions were made: 2 mM ATP and 300 nM GroES (*open square*) and 2 mM ATP, 300 nM GroES, and 5 mM mizoribine (*closed circle*). For the spontaneous folding reaction (*open triangle*), denatured citrate synthase was diluted directly into the buffer and pre-equilibrated to 35 °C.

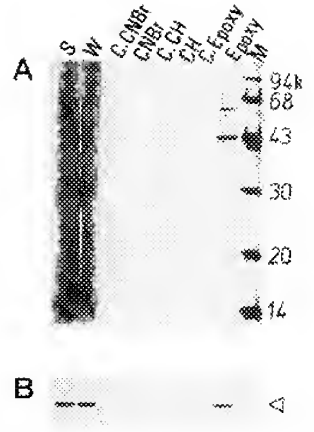

FIG. 8. **Mizoribine-binding proteins using different types of mizoribine affinity column chromatography.** Mizoribine affinity columns were prepared using different affinity matrices (CNBr-activated Sepharose 4B, activated CH-Sepharose 4B, or activated epoxy-Sepharose 6B). Porcine kidney cytosols were applied onto these columns, washed with buffer containing 0.15 M NaCl, and the binding proteins were washed with 10 mM mizoribine. The eluants were analyzed by SDS-PAGE (12.5% gel), stained with 0.1% Coomassie Brilliant Blue (v/v), and destained with 25% isopropyl alcohol (v/v) and either 10% acetic acid (v/v) (*A*) or by immunoblotting using anti-HSP60 antibody (*B*). In *panels A* and *B*, *S*, *W*, and *M* denote applied samples, washed proteins, and molecular standard proteins, respectively. In *panel A*, *C.CNBr*, *C.CH*, and *C.Epoxy* denote control affinity gels of the matrices (CNBr-activated Sepharose 4B, activated CH-Sepharose 4B, or activated epoxy-Sepharose 6B not coupled with mizoribine, respectively). *CNBr*, *CH*, and *Epoxy* denote the mizoribine affinity column prepared with CNBr-activated Sepharose 4B, activated CH-Sepharose 4B, or activated epoxy-Sepharose 6B, respectively.

DISCUSSION

Mizoribine has been used clinically as an immunosuppressant. This agent is a competitive inhibitor of inosine-monophosphate dehydrogenase (IMPDH) and acts to inhibit *de novo* purine synthesis by inhibition of IMPDH (22). Mizoribine also inhibits GMP synthase (11). The inhibition of these enzymes

results in the depletion of the intracellular guanine nucleotide. The depletion of the guanine nucleotide causes $G_{1/S}$ blockage in human T cells (12). Although immunophilins have been investigated for immunosuppressants cyclosporin A, FK506, rapamycin, and deoxyspergualin, immunophilin specific for mizoribine has not yet been investigated. Molecular chaperones HSP70 or HSP90 associate with the immunophilins of cyclosporin A or FK506 and/or rapamycin (23). Both molecular chaperones bind directly to immunosuppressant deoxyspergualin (4, 5). HSP70 and/or HSP90 interact with the immunosuppressant as directly or indirectly as does immunophilin. Among the molecular chaperones, only these two HSPs have been reported as immunophilin or immunophilin-associated proteins, but there have been no reports that the other molecular chaperones, including HSP60, act as immunophilins or their binding proteins.

In the present study, we have identified two mizoribine-binding proteins, HSP60 and actin, by using a mizoribine affinity column. Through affinity column chromatography and surface plasmon resonance analysis we could confirm that HSP60 has high affinity to mizoribine. HSP60 recognized the NH_2 group of the agent. The binding properties of HSP60 to mizoribine are the same to immunosuppressant deoxyspergualin as those of HSP70 and HSP90, because these proteins also bind to the agent directly and may act as immunophilins in the cells. The binding of mizoribine to HSP60 resulted in the interference of chaperone activity of HSP60 *in vitro*. It has been reported that mammalian mt-cpn60 is only able to interact with mammalian mt-cpn10 in contrast with other species of cpn10 (24). There is a discrepant result in an *in vitro* folding assay. We used wild type HSP60 that was purified from porcine liver cytosol in the assay (13). One possibility for achieving the discrepant results may be the difference between recombinant mt-cpn60 and wild type HSP60. This difference may result in discrepancies during *in vitro* folding assay. These results indicated that the interaction between HSP60 and mizoribine may be significant.

Prokaryotic chaperonins (such as HSP60) have been well characterized (25, 26), and the crystal structure of the asymmetric GroEL-GroES-$(ADP)_7$ chaperonin complex has been shown by Xu *et al.* (27). The bacterial chaperonin GroEL and its co-chaperonin GroES are coexpressed from a common operon (*GroE*) in *Escherichia coli*. GroEL contains 14 identical subunits of relative molecular weight of 58,000 that are assembled as two heptameric rings stacked back to back. GroES contains seven identical 10,000-dalton subunits assembled as one heptameric ring. Both chaperonins are essential for protein folding under all cell conditions. Chaperonins seem to provide kinetic assistance to the process of folding newly translated or newly translocated polypeptides. Conversely, difficult purification of mammalian HSP60 results in few reports being made about the protein.

Mammalian HSP60 was first cloned and sequenced by Guputa and co-workers (20) as a mitochondrial P1 protein. The deduced amino acid sequence of P1 shows strong homology to GroEL and the 65-kDa major antigen of mycobacteria. It has been shown that the possibility of involvement of the P1 protein in certain autoimmune diseases is because of high homology between P1 and the mycobacterial antigen (20). We have purified and identified mammalian HSP60 from porcine liver cytosol or porcine kidney mitochondria (13). Although both proteins exhibit molecular chaperone activity *in vitro*, there were some differences between cytoplasmic HSP60 and mitochondrial HSP60; the former has a signal sequence in the NH_2 terminus of the protein (1–26 amino acid residues), but the latter does not. The protein is localized in cytoplasm, mitochondria, and nuclei during immunoblotting or electron microscopic immunohistochemistry (13). The functional HSP60 in the cytoplasm may be transported into the mitochondria, and the protein may be processed to mitochondrial HSP60 in the organelle.

The physiological functions of mammalian HSP60 are not yet fully understood. Important physiological roles of the proposed HSP60 have been raised. It has been reported that HSP60 associates with $p21^{ras}$ (28) and that the protein is a major target for modification during *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine-induced nephrotoxicity (29). We have shown here that HSP60 binds directly to the immunosuppressant and that the protein may act as an immunophilin. These findings represent the first demonstration of HSP60 as an immunophilin. Further studies are needed to clarify the physiological implications of the binding of HSP60 to mizoribine. As mentioned above, mizoribine is a competitive inhibitor of IMPDH and acts to inhibit *de novo* purine synthesis by inhibition of IMDPH (22). Recently, it has been reported that human but not bacterial HSP60 can contribute to suppression of arthritis by the stimulation of regulatory suppressive T cell activity (30). Mizoribine has been used clinically as an immunosuppressant for the treatment of rheumatoid arthritis (31). The binding of HSP60 to mizoribine may enhance the inhibitory activity of IMPDH or stimulate regulatory suppressive T cell activity *in vivo*.

REFERENCES

1. Galat, A. (1993) *Eur. J. Biochem.* **216,** 689–707
2. Fruman, D. A., Burakoff, S. J., and Bière, B. E. (1994) *FASEB J.* **8,** 391–400
3. Tai, P-K. K., Albers, M. W., Chang, H., Faber, L. E., and Schreiber, S. L. (1992) *Science* **256,** 1315–1318
4. Nadler, S. G., Tepper, M. A., Schacter, B., and Mazzucco, C. E. (1992) *Science* **258,** 484–486
5. Nadeau, K., Nadler, S. G., Saulnier, M., Tepper, M. A., and Walsh, C. T. (1994) *Biochemistry* **33,** 2561–2567
6. Mizuno, K., Tsujino, M., Takada, M., Hayashi, M., Atsumi, K., Asano, K., and Matsuda, T. (1974) *J. Antibiot. (Tokyo)* **27,** 775–782
7. Fukui, M., Inaba, M., Tsukagoshi, S., and Sakurai, Y. (1982) *Cancer Res.* **42,** 1098–1102
8. Tajima, A., Hata, M., Ohtani, N., Ohtawara, Y., Suzuki, K., and Aso, Y. (1984) *Transplantation* **38,** 116–118
9. Nakajima, A., Kanai, A., Minami, S., and Kogure, M. (1985) *Am. J. Ophtalmol.* **100,** 161–163
10. Koyama, H., and Tsuji, M. (1983) *Biochem. Pharmacol.* **32,** 3547–3553
11. Kusumi, T., Tsuda, M., Katsumata, T., and Yamamura, M. (1989) *Cell Biochem. Funct.* **7,** 201–204
12. Turka, L. A., Dayton, J., Sinclair, G., Thompson, C. B., and Mitchell, B. S. (1991) *J. Clin. Invest.* **87,** 940–948
13. Itoh, H., Kobayashi, R., Wakui, H., Komatsuda, A., Ohtani, H., Miura, A. B., Otaka, M., Masamune, O., Andoh, H., Koyama, Y., Sato, Y., and Tashima, Y. (1995) *J. Biol. Chem.* **270,** 13429–13435
14. Itoh, H. and Tashima, Y. (1993) *Int. J. Biochem.* **25,** 69–77
15. Laemmli, U. K. (1970) *Nature* **227,** 680–685
16. Towbin, H., Staephlin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 4350–4354
17. Itoh, H., and Tashima, Y. (1997) *Biochem. J.* **326,** 567–572
18. Buchner, J., Schmidt, M., Fuchs, M., Jaenicke, R., Rudolph, R., Schmidt, F. X., and Kiefhaber, T. (1991) *Biochemistry* **30,** 1586–1591
19. Schmidt, M., Buchner, J., Todd, M. J., Lorimer, G. H., and Viitanen, P. V. (1994) *J. Biol. Chem.* **269,** 10304–10311
20. Jindal, S., Dudani, A. K., Singh, B., Harley, C. B., and Guputa, R. S. (1989) *Mol. Cell. Biol.* **9,** 2279–2283
21. Erba, H. P., Eddy, R., Shows, T., Kedes, L., and Gunning, P. (1988) *Mol. Cell. Biol.* **8,** 1775–1789
22. Halloran, P. F. (1996) *Clin. Transplant.* **10,** 118–123
23. Moss, M. L., Palmer, P. E., Kuzmic, P., Dunlap, B. E., Henzel, W., Kofron, J. L., Mellon, W. S., Royer, C. E., and Ritch, D. H. (1992) *J. Biol. Chem.* **267,** 22054–22059
24. Viitanen, P. V., Lorimer, G. H., Bergmeier, W., Weiss, C., Kessel, M., and Goloubinoff, P. (1998) *Methods Enzymol.* **290,** 203–230
25. Hartl, F. U. (1996) *Nature* **381,** 571–580
26. Fenton, W. A., and Horwich, A. L. (1997) *Protein Sci.* **6,** 743–760
27. Xu, Z., Horwich, A. L., and Sigler, P. B. (1997) *Nature* **388,** 741–750
28. Ikawa, S., and Weinberg, R. A., (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 2012–2016
29. Bruschi, S. A., West, K. A., Crabb, J. W., Guputa, R. S., and Stevens, J. L. (1993) *J. Biol. Chem.* **268,** 23157–23161
30. van Roon, J. A., van Eden, W., van Roy, J. L., Lafeber, F. J, and Bijlsma, J. W. (1997) *J. Clin. Invest.* **100,** 459–463
31. Shiokawa, Y., Honma, M., Shichikawa, K., Miyamoto, T., Hirose, S., Nobunaga, T., Mizushima, Y., Sugawara, S., Warabi, H., Kondo, H., and Ogawa, H. (1991) *Ensho* **11,** 375–396